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(54) Title: PROCESS FOR THE PREPARATION OF HYPOALLERGENIC MOSAIC ANTIGENS

(57) Abstract: A process for the preparation of an hypoallergenic mosaic antigen derived from an allergen is disclosed whereby a) in a first step the allergen is split into at least two parts and the IgE reactivity of each part is determined and b) in a second step those parts of the allergen which have no detectable IgE reaction are combined to a mosaic antigen which comprises the amino acids of the allergen but the order of the amino acids of the mosaic antigen is different from that of the naturally occurring allergen.

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Process for the preparation of hypoallergenic mosaic antigens

The present invention relates to mosaic antigens derived from naturally occurring allergens, in particular timothy grass pollen allergen Phl p 2. The mosaic antigens display reduced allergenic activity and are useful as allergy vaccines for the treatment of sensitized allergic patients and for prophylactic vaccination.

A large percentage of the population suffers from IgE-mediated allergies. Those patients suffer from allergic reactions against several antigens. A high percentage of the allergic reactions are caused by plant pollen. The symptoms of allergy like allergic rhinoconjunctivitis, asthma, dermatitis and even anaphylactic shock are due to IgE recognition of allergens. The IgE molecules are largely responsible for the symptoms of allergic reactions such as hay fever, asthma and hives.

The IgE molecules bind to an allergen like e.g. plant pollen. The tail region of the IgE molecule, the Fc part binds to Fc receptors which are mainly located on the surface of mast cells in tissues and basophils in the blood. Antigen binding triggers the mast cells or basophils to secrete a variety of cytokines and biologically active compounds, especially

The term "hypoallergenic mosaic antigen" provided by the present process means that the antigen comprises substantially all amino acids of the naturally occurring allergen. The difference compared with the naturally occurring antigen is, however, that the allergen is in a first step split into different parts. When the amino acid sequence of the allergen is known it is common general knowledge of a person skilled in the art to prepare peptides of varying lengths from the antigen. The peptides may either be prepared by chemical synthesis which is well-known in the art. Alternatively the peptides can be easily prepared by Polymerase Chain Reaction since suitable primers can be easily synthesized when the sequence is known.

The reactivity of each part of the allergen which is present as a peptide or polypeptide has to be determined. This can be done by reacting the peptide with sera from patients which are allergic against the naturally occurring allergen. The IgE antibodies present in such sera will react with the peptide if an IgE epitope is present on the peptide. If there are, however, no linear IgE epitopes or if conformational IgE epitopes are destroyed by separating the whole naturally occurring allergen there will be no binding of IgE with the peptide. The IgE antibodies can subsequently easily be detected by reaction with specific anti-antibodies which bind to the IgE antibody. Those anti-antibodies are usually labeled for detection.

It is an important aspect of the present invention to divide the allergen into such parts which do not react with IgE antibodies. If a part of the allergen still reacts with IgE antibodies in a substantial amount such parts of the allergen should not be used for the preparation of the mosaic antigen. It is advisable to test the parts of the naturally occurring antigen to be used in the mosaic antigen with sera from different allergic patients since there may be variations with regard to specifity and amount of IgE concentration in each serum.

When the allergen has been split up into several parts which do not have any detectable IgE reactivity those parts are newly arranged in order to provide the mosaic antigen. That the part of the allergen does not have a substantial IgE reactivity means that the IgE reactivity of the whole, naturally occurring allergen is tested with preferably at least five sera from allergic patients and the parts thereof are tested as well. The binding of IgE molecules to the allergen and the parts thereof is determined quantitatively and the IgE reactivity of the part has to be reduced to not more than 10%, preferably not more than 5% of the value obtained for the naturally occurring allergen.

exposure and correction of seasonally elevated antibody levels to basal values. J Allergy Clin Immunol 1987, 80, 646-655. Ansari AA, Shenbagamurthi P, Marsh DG. Complete amino acid sequence of a Lolium perenne (perennial rye grass) pollen allergen, Lol p II. J Biol Chem 1989, 264, 11181-11185. Dolecek C, Vrtala S, Laffer S, Steinberger P, Kraft D, Scheiner O, Valenta R. Molecular characterization of Phl p II, a major timothy grass (Phleum pratense) pollen allergen. FEBS Lett 1993, 335, 299-304.

In an especially preferred embodiment the allergen used for the mosaic antigen is the timothy grass pollen allergen Phl p 2. The sequence of the grass pollen allergen Phl p 2 is disclosed in WO 94/23035. A more detailed description of the Phl p 2 from timothy grass pollen is provided in De Marino et al., Structure (1999) Vol. 7, No. 8, p. 943-952. The Phl p 2 antigen is preferred since it reacts with serum IgE from about 70% of grass pollen allergic individuals and elicits histamine release from basophils of sensitized patients.

In the course of the present invention it has been found that the Phl p 2 allergen is preferably split into three peptides, namely peptide 1 having amino acids 1-33, peptide 2 having amino acids 34-64 and peptide 3 having amino acids 65-96. By rearranging the peptides in the order 1, 3 and 2 a mosaic antigen is provided which can be used for hypoallergenic vaccination. This mosaic antigen has the advantage that a sufficient amount of blocking IgE antibodies is produced, but the undesired side-reactions associated with the vaccination are nearly completely avoided.

The amino acid sequence of the preferred mosaic allergen has SEQ ID NO:1. The DNA coding for this preferred mosaic allergen has SEQ ID NO:2.

The mosaic allergen provided by the present disclosure can preferably be used for the preparation of a medicament for the treatment of an allergic reaction. The preferred Phl p 2 mosaic antigen can be used for the preparation of a medicament for the treatment of grass pollen allergy. Since the Phl p 2 is an antigen against which a large percentage of allergic patients have formed IgE antibodies the mosaic antigen is very helpful in the treatment of patients suffering from hay fever.

Figure 3: DNA sequence of the primers used for the construction of the Phl p 2 mosaic and schematic representation of PCR approach used for the assembly of the cDNA coding for the rPhl p 2 mosaic. The Nde I and Eco R I restriction sites are underlined in primer P2/1 and P2/6, respectively. The primers correspond to SEQ ID NO:6 to SEQ ID NO:11.

Figure 4: cDNA (SEQ ID NO:2) and deduced amino acid sequence (SEQ ID NO:1) and of the his-tagged Phl p 2 mosaic. Aminoacids are displayed in the single letter code, base pair and amino acid numbers are shown on the right margin.

Figure 5: Purity of rPhl p 2 mosaic and rPhl p 2. Comassie stained gel containing Phl p 2 (lane P2), Phl p 2 mosaic (lane P2M) and a molecular weight marker (lane M).

Figure 6: Mass spectroscopical analysis of purified rPhl p 2 mosaic (A) and rPhl p 2 (B). The mass/charge ratio is shown on the x-axis and the signal intensity is expressed as percentage of the most intensive signal obtained in the investigated mass range.

Figure 7: Comparison of the IgE binding capacity of rPhl p 2 (P2) and the rPhl p 2 mosaic (P2M). Nitrocellulose dotted rPhl p 2 (P2) and rPhl p 2 mosaic (P2M), as well as human serum albumine (HSA) were probed with serum from 12 Phl p 2-reactive grass pollen allergic patients (1-12). Bound IgE antibodies were detected with ^{125}I-labeled anti-human IgE antibodies and visualized by autoradiography.

Figure 8: Reduced allergenic activity of rPhl p 2 mosaic determined by basophil histamine release. Basophils from a grass pollen allergic patient were exposed to increasing concentrations of rPhl p 2 and rPhl p 2 mosaic (x-axis). Histamine release is expressed as percentage of total histamine release on the y-axis.

Figure 9: Rabbit anti-rPhl p 2 mosaic antibodies recognize the rPhl p 2 wildtype allergen. Rabbit antisera raised against the rPhl p 2 mosaic (αP2M), KLH-coupled mosaic (αP2M-KLH) and rPhl p 2 (αPhl p 2) as well as buffer (C) were exposed to dot-blotted KLH, human serum albumin (HSA), rPhl p 2 (P2) and rPhl p 2 mosaic (P2M). Bound rabbit antibodies were detected with ^{125}I-labeled donkey anti-rabbit IgG and visualized by autoradiography.

peptides was checked by mass-spectrometry and they were purified to >90% purity by preparative HPLC (PiChem, Graz, Austria) (Focke M, Mahler V, Ball T, Sperr WR, Majlesi Y, Valent P, Kraft D, Valenta R. Nonanaphylactic synthetic peptides derived from B cell epitopes of the major grass pollen allergen, Phl p 1, for allergy vaccination. FASEB J. 2001, 15: 2042-2044.

The allergenic activity of the Phl p 2-derived peptides was evaluated by comparing the IgE-reactivity of complete rPhl p 2 with the peptides by dot blot analysis (Figure 1). Nitrocellulose-dotted Phl p 2-derived peptides (P1-P3), an immunologically unrelated major grass pollen allergen, rPhl p 5 (Vrtala S, Sperr WR, Reimitzer I, van Ree R, Laffer S, Müller WD, Valent P, Lechner K, Rumpold H, Kraft D, Scheiner O, Valenta R. cDNA cloning of a major allergen from timothy grass (Phleum pratense) pollen; characterization of the recombiniant Phl p V allergen. J.Immunol. 1993, 151: 4773-4781), and for control purposes, human serum albumin as well as a control peptide were exposed to sera from grass pollen allergic patients and to serum from a non-allergic individual.

Bound IgE antibodies were detected as described previously (Valenta R, Duchêne M, Ebner C, Valent P, Sillaber C, Deviller P, Ferreira F, Tejkl M, Edelmann H, Kraft D, Scheiner O. Profilins constitute a novel family of functional plant pan-allergens. J.Exp.Med. 1992, 175: 377-385). Sera from all 35 grass pollen allergic patients showed IgE reactivity to nitrocellulose-dotted rPhl p 2 but no serum reacted with any of the three Phl p 2-derived peptides (Figure 1). Serum from the non-allergic individual displayed no IgE reactivity to any of the peptides or proteins.

Example 2: Characterization of the recombinant Phl p 2 mosaic protein.

A recombinant Phl p 2 mosaic protein was obtained by recombination of the three Phl p 2-derived peptides in altered sequence. This mosaic protein was created under the assumption that recombination of three non-allergenic Phl p 2 fragments in altered order will deliver a mosaic protein with disrupted three-dimensional structure and consequently reduced allergenic activity. In addition it was expected that the mosaic protein will exhibit better immunogenicity compared to the individual smaller peptide units and preserve the entire primary amino acid sequence of Phl p 2 thus containing the relevant T cell epitopes of Phl p 2.

advice (Quiagen, Hilden, Germany). Protein samples were analysed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and protein staining (Fling SP, Gregerson DS. Peptide and protein molecular weight determination by electrophoresis using a high-molarity Tris buffer system without urea. Anal.Biochem. 1986, 155:83-88) (Figure 4).

Figure 5 shows the purity of the his-tagged recombinant proteins (rPhl p 2: P2; rPhl p 2 mosaic: P2M). Although the two proteins did not show a completely identical migration behaviour in the SDS-PAGE, mass spectroscopical analysis performed as described by Niederberger V, Hayek B, Vrtala S, Laffer S, Twardosz A, Vangelista L, Sperr WR, Valent P, Rumpold H, Kraft D, Ehrenberger K, Valenta R, Spitzauer S. Calcium-dependent immunoglobulin E recognition of the apo- and calcium-bound form of a cross-reactive two EF-hand timothy grass pollen allergen, Phl p 7. FASEB J. 1999, 13: 843-856 showed almost identical molecular weights of the two proteins (rPhl p 2: 11775 Da; rPhl p 2 mosaic: 11770 Da) which were in good agreement with the deduced molecular weights including the methionines at their N-terminus (Figure 6).

Example 3: rPhl p 2 mosaic lacks IgE reactivity and allergenic properties

The IgE binding capacity of purified Phl p 2 mosaic (P2M) was compared with that of Phl p 2 wildtype by dot blot experiments as described for the peptides using sera from twelve timothy grass pollen allergic patients (Figure 7). Sera from all 12 grass pollen allergic patients contained IgE antibodies against rPhl p 2 but no serum exhibited IgE reactivity to the rPhl p 2 mosaic or the negative control, human serum albumin (Figure 7). The strongly reduced allergenic activity of the rPhl p 2 mosaic was further demonstrated by basophil histamine release and skin test experiments. Basophils from a grass pollen allergic patient were enriched by dextran sedimentation and exposed to increasing concentrations of purified rPhl p 2 or rPhl p 2 mosaic as described (Valent P, Besemer J, Muhm M, Majdic O; Lechner K, Bettelhei P. Interleukin 3 activates human blood basophils via high-affinity binding sites. Proc.Natl.Acad.Sci. USA 1989, 86: 5542-5546).

Histamine released in the cell free supernatants was determined in triplicates by radioimmuno assay and is expressed as mean percentage of the total histamine content of the cells as described by Valent *et al.*

The rabbit anti-rPhl p 2 mosaic antiserum reacted strongly with the immunogen (rPhl p 2 mosaic) as well as with the rPhl p 2 allergen (Figure 9). The antibody reactivity was of comparable intensity as that obtained with the antiserum produced by immunization with the KLH-coupled mosaic and stronger than the reactivity induced by immunization with the rPhl p 2 allergen (Figure 9).

Example 6: Measurement of blocking antibodies

It was studied whether IgG antibodies induced by immunization with the rPhl p 2 mosaic inhibit the binding of allergic patients' serum IgE to complete rPhl p 2 by ELISA competition using sera from five grass pollen allergic patients (Table 3). ELISA plates (Nunc Maxisorp, Rokslide, Denmark) were coated with rPhl p 2 (1µg/ml) and preincubated either with a 1:100 dilution of each of the anti-Phl p 2 mosaic and anti-Phl p 2 antiserum and, for control purposes, with the corresponding preimmunsera. After washing plates were incubated with 1:3 diluted sera from five Phl p 2-sensitized grass pollen allergic patients and bound IgE antibodies were detected with alkaline phosphate conjugated monoclonal rat anti-human IgE antibody (Pharmingen, San Diego, CA), diluted 1:1000. The percentage inhibition of IgE binding achieved by preincubation with the anti- Phl p 2 mosaic and Phl p 2 was calculated as follows: %inhibition of IgE binding= $100\text{-}OD_I/OD_P \times 100$. OD_I and OD_P represent the extinctions after preincubation with the rabbits immune and preimmune serum, respectively as described by Focke *et al.*, 2001.

The anti-Phl p 2 mosaic antibodies inhibited the binding of grass pollen allergic patients IgE binding to Phl p 2 (20.93% average inhibition) albeit to a lower degree as was achieved by preincubation with antibodies induced by immunization with the rPhl p 2 allergen (54.73% average inhibition).

The results of the immunization studies thus show that antibodies raised against the rPhl p 2 mosaic recognize the Phl p 2 wildtype allergen and inhibit allergic patients IgE recognition of Phl p 2.

6) Process according to claim 5 wherein the allergen Phl p 2 is split into three peptides, namely peptide 1 having amino acids 1-33, peptide 2 having amino acids 34-64 and peptide 3 having amino acids 65-96 of the amino acid sequence of naturally occurring Phl p 2 and the mosaic antigen is provided by linking the peptides in the order peptide 1, peptide 3 and peptide 2.

7) Mosaic allergen having the amino acid sequence of SEQ ID NO:1.

8) DNA sequence having SEQ ID NO:2 coding for the mosaic allergen of claim 7 or a sequence complementary thereto.

9) Use of a mosaic allergen obtainable by a process according to any one of claims 1-6 for the preparation of a medicament for the treatment of an allergic reaction.

10) Use of a mosaic allergen according to claim 9 wherein the allergic reaction is caused by grass pollen.

11) Use of a mosaic allergen according to claim 10 wherein the allergic reaction is caused by timothy grass pollen.

12) Use of a mosaic allergen according to claim 11 wherein the allergic reaction is caused by timothy grass pollen Phl p 2.

13) Vaccine for the treatment of allergic patients characterized in that it comprises a mosaic allergen obtainable by any process according to claims 1-6 or the mosaic allergen of claim 7.

14) Vaccine for the treatment of grass pollen allergic patients characterized in that it comprises a DNA sequence coding for a mosaic antigen obtainable by a process according to any of claims 1-6 or the DNA of claim 8 or sequences complementary to any of these DNA sequences.

Table 1.

	Position aa	Sequence	Number of aa	Molecular weight	Isoelectric point
Peptide 1	1-33	VPKVTFTVEKGSNEKHLAVLVKYEGDTMAEVELC	34	3765,02	4.87
Peptide 3	65-96	REHGSDEWVAMTKGEGGVWTFDSEEPLQGPFNC	33	3696,8	4.03
Peptide 2	34-64	CFRFLTEKGMKNVFDDVVPEKYTIGATYAPEE	32	3698,91	4.45

Table 2.

Induction of immediate skin reactions with Phl p 2 and Phl p 2 mosaic

Individual 1

	mean wheal diameter (mm)	
	P2	P2M
1 µg/ml	9	0
2 µg/ml	10	0
4 µg/ml	11	0
8 µg/ml	14	5
16 µg/ml	10	4
Timothy grass	15	
Histamine	8	

Individual 2

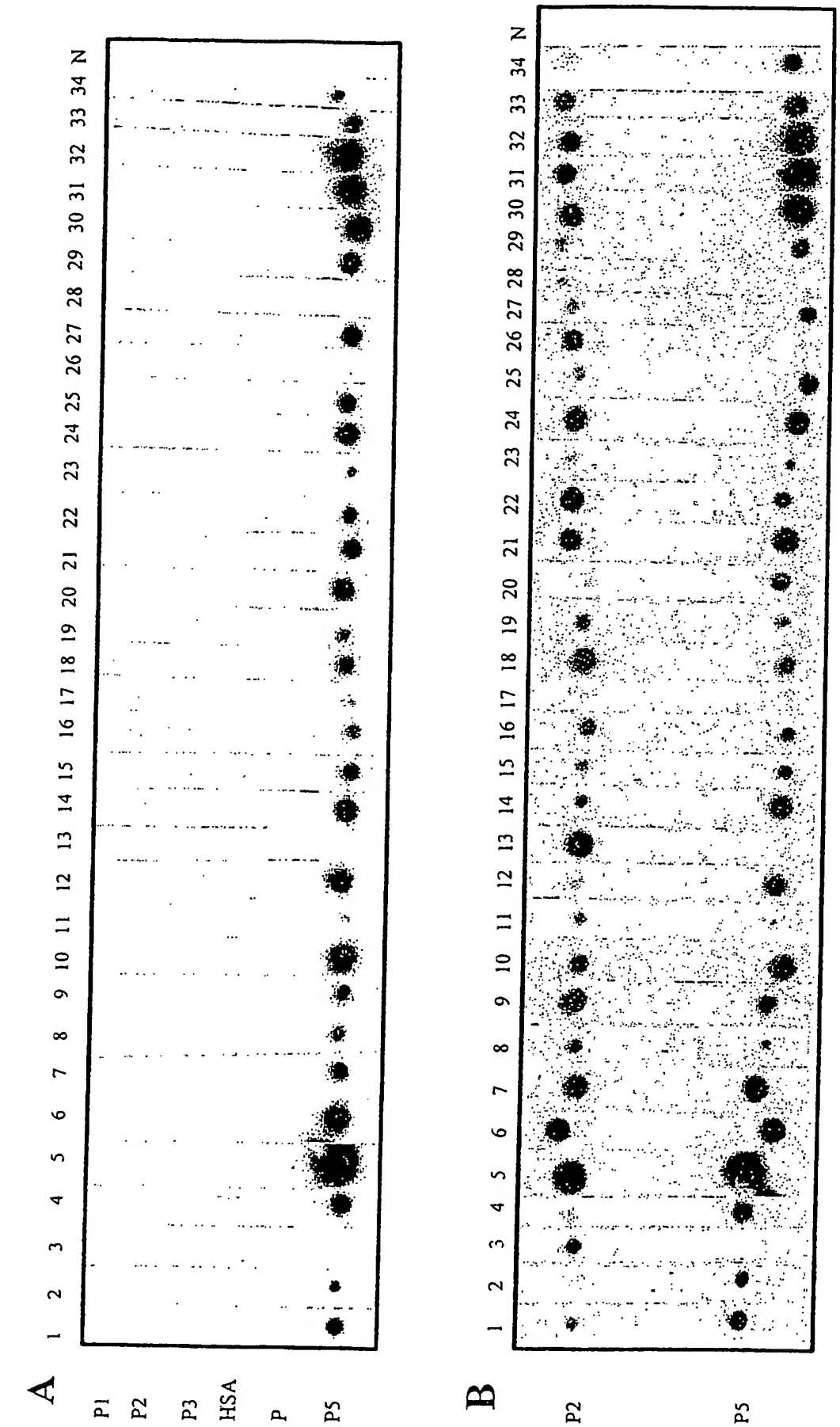
	mean wheal diameter (mm)	
	P2	P2M
1 µg/ml	5	0
2 µg/ml	5	0
4 µg/ml	6	0
8 µg/ml	7	0
16 µg/ml	9	3
Timothy grass	10	
Histamine	8	

Table 3.

Rabbit anti P2 mosaic and rabbit anti-rPhl p 2 antibodies inhibit IgE binding of grass pollen allergic patients to rPhl p 2

	% inhibition	
Patient	**anti-P2M**	**anti-rPhl p 2**
1	**39.54**	**76.93**
2	**9.25**	**63.97**
3	**23.20**	**52.91**
4	**14.38**	**40.86**
5	**18.27**	**38.97**
Mean	**20.93**	**54.73**

Figure 1.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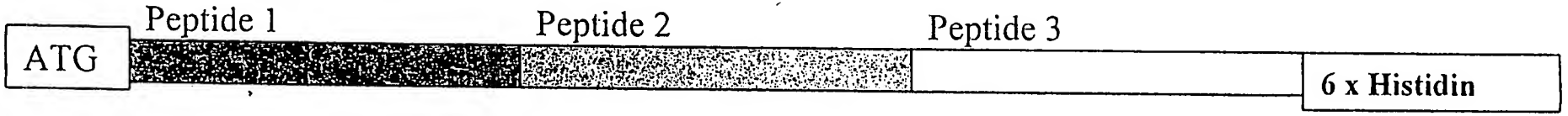
P1
P2
P3
HSA
P
P5
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 N
P2
P5

Figure 2.

P 2



P 2 mosaic

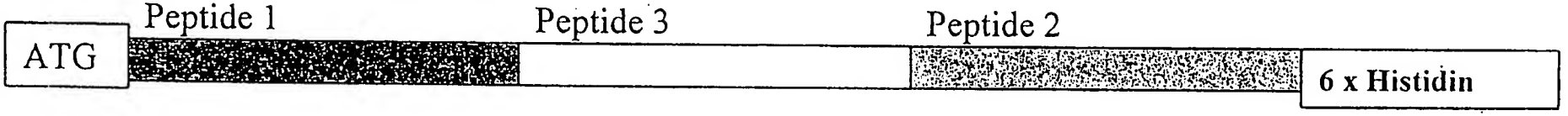


Figure 3.

SEQ ID NO:

6 P2/1: 5′ - GGA TTT CCA TAT GGT CCC GAA GGT GAC GTT CAC G - 3′

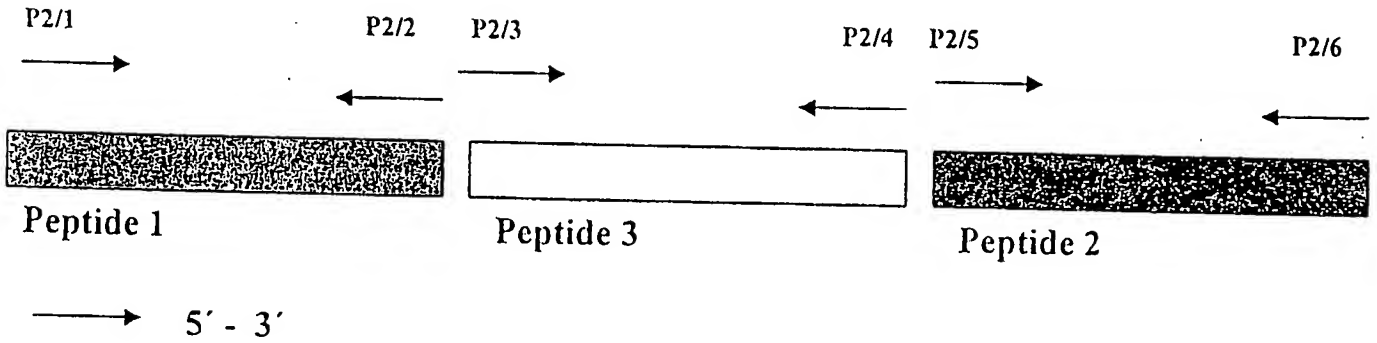
7 P2/2: 5′ - GGT GAG GAA CCG GAA GAG CTC CAC CTC CGC CAT GGT - 3′

8 P2/3: 5′ - GCG GAG GTG GAG CTC TTC CGG TTC CTC ACC GAG AAG - 3′

9 P2/4: 5′ - GGA GCC GTG CTC CCG CTC TTC TGG CGC GTA GGT GGC - 3′

10 P2/5: 5′ - TAC GCG CCA GAA GAG CGG GAG CAC GGC TCC GAC GAG - 3′

11 P2/6: 5′ - CGC GAA TTC TCA GTG GTG GTG GTG GTG GTG GTT GAA GGG CCC CTG GAG CGG - 3′



10/542735
PCT/EP2003/014507

Figure 4.

P2M-Sequence

ATG GTC CCG AAG GTG ACG TTC ACG GTG GAG AAG GGG TCC AAC GAG AAG CAC	51
M V P K V T F T V E K G S N E K H	17
CTG GCG GTG CTG GTG AAG TAC GAG GGG GAC ACC ATG GCG GAG GTG GAG CTC	102
L A V L V K Y E G D T M A E V E L	34
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F R F L T E K G M K N V F D D V V	51
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P E K Y T I G A T Y A P E E R E H	68
GGC TCC GAC GAG TGG GTC GCC ATG ACC AAG GGG GAG GGC GGC GTG TGG ACG	255
G S D E W V A M T K G E G G V W T	85
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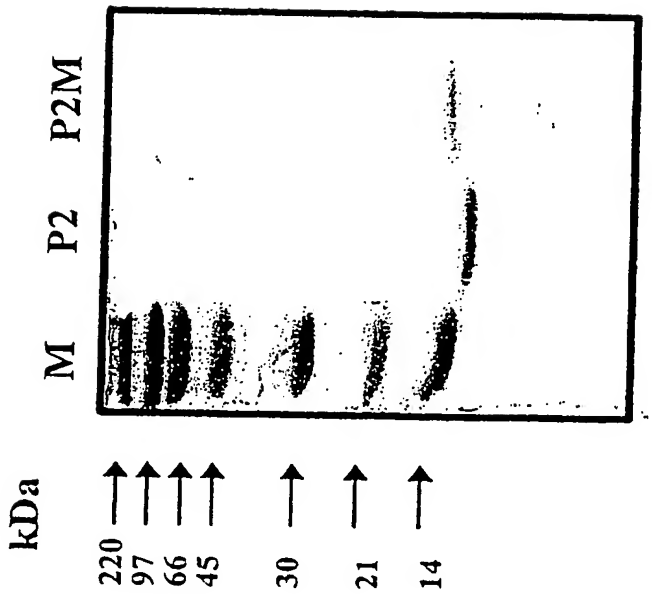
H	103



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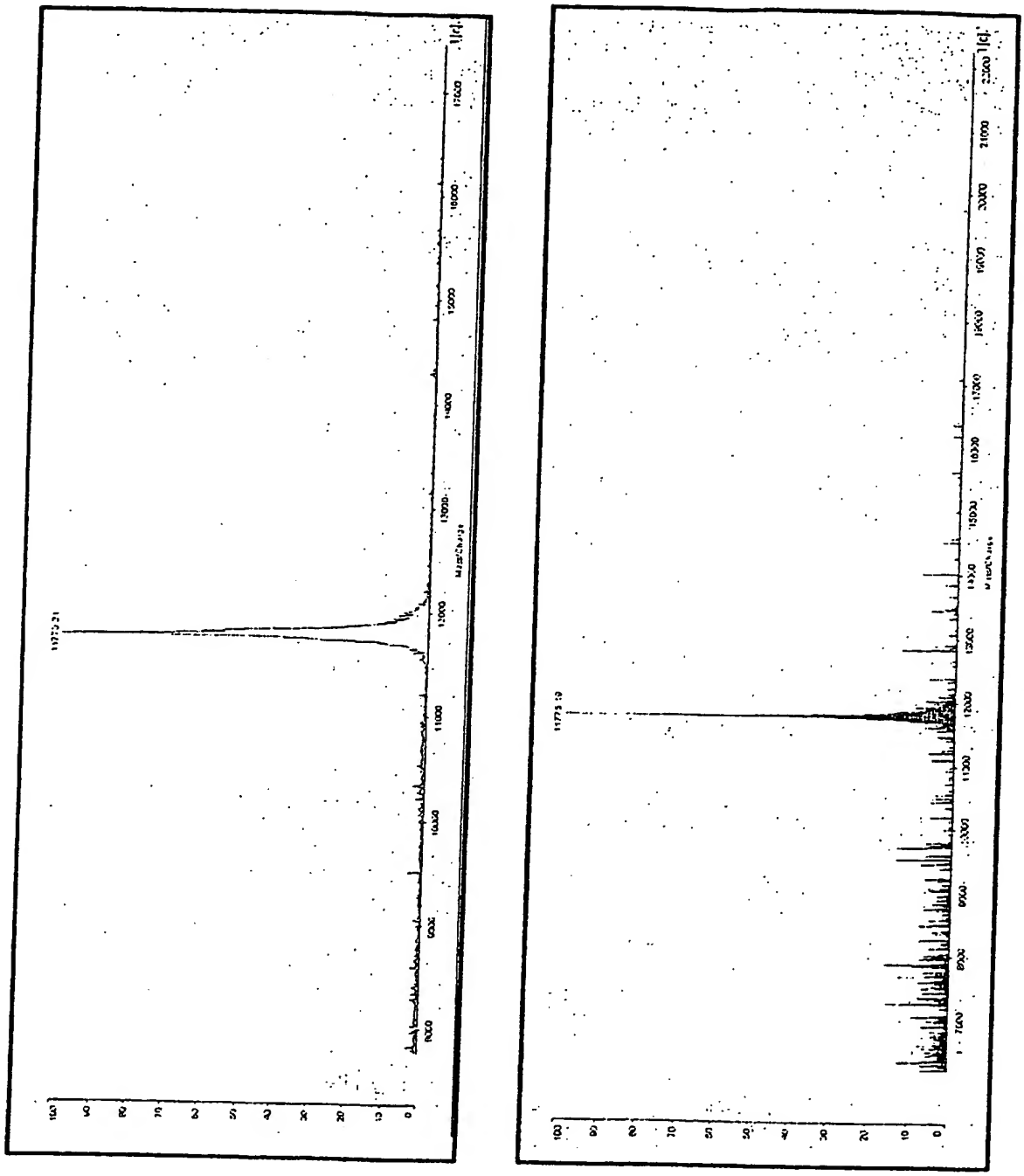
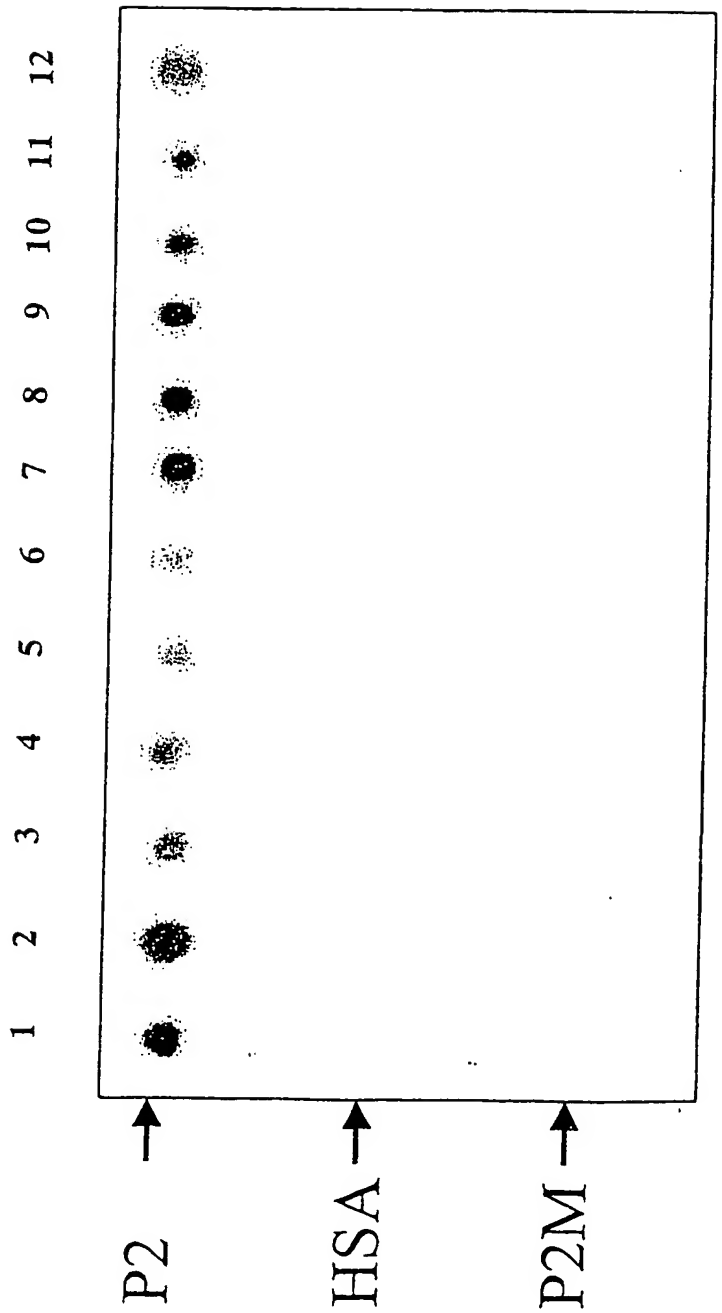
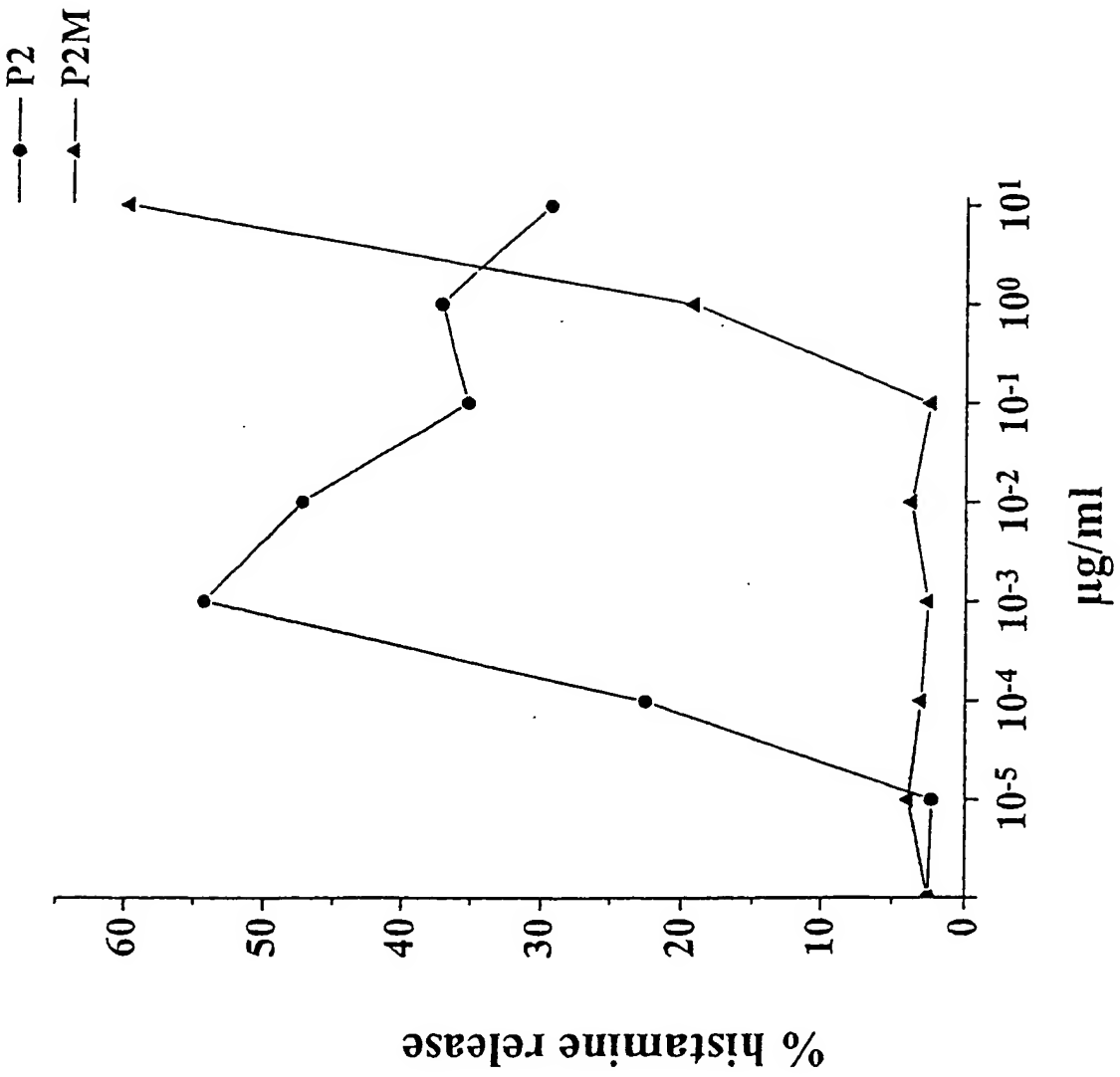


A B

Figure 6

Figure 7.

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20
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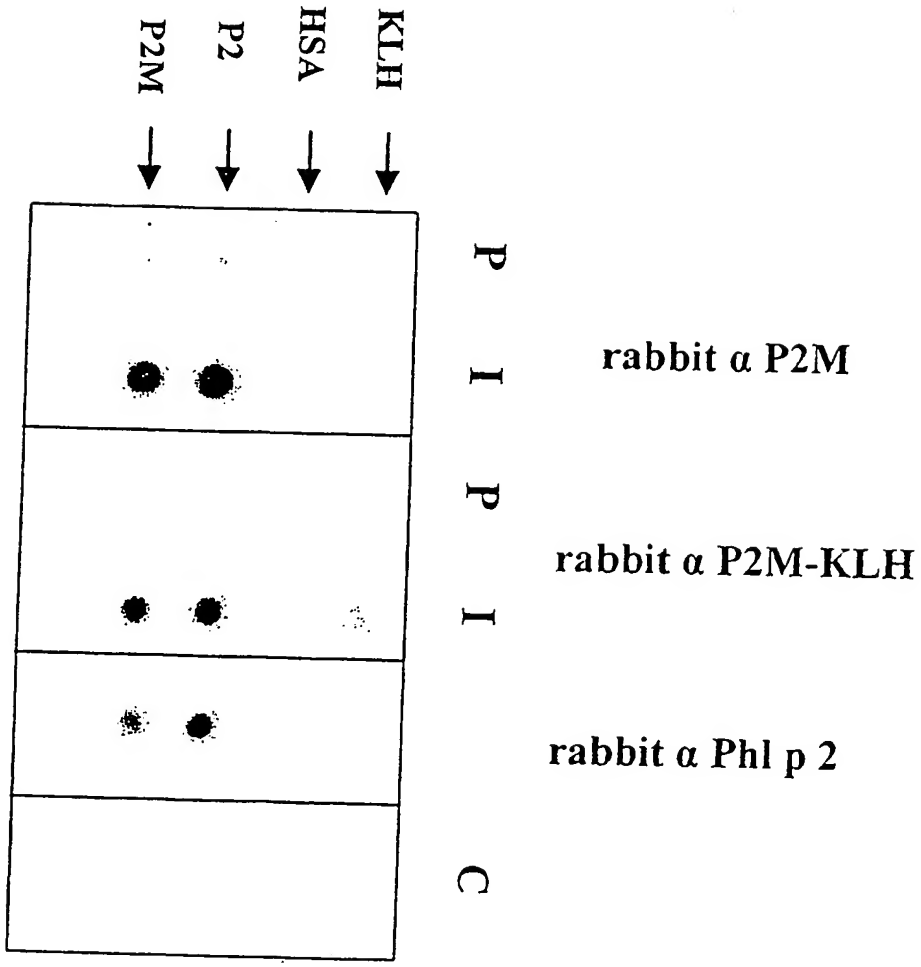
40
50
60
10-5
10-4
10-3
10-2
10-1
100
101
µg/ml

Figure 8.


P2M
P2
HSA
KLH
P I P I C
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rabbit α Phl p 2

Figure 9.

SEQUENCE LISTING

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Asn His His His His His His
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<223> Description of Artificial Sequence: primer

<400> 6
ggatttccat atggtcccga aggtgacgtt cacg 34

<210> 7
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 7

cgcgaattct cagtggtggt ggtggtggtg gttgaagggc ccctggagcg g 51

<210> 12
<211> 51
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 12
cgcgaattct cagtggtggt ggtggtggtg ctcttctggc gcgtaggtgg c 51